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With international search report.

(54) Title: LIPID A ANALOG/IMMUNOGENIC CARRIER CONJUGATES AND THE USE THEREOF AS VACCINES

(57) Abstract

The invention relates to a lipid A analog/immunogenic carrier conjugates and the use thereof as vaccines for the prophylaxis of septic shock in animals.

* See back of page

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Title of the Invention

LIPID A ANALOG/IMMUNOGENIC CARRIER CONJUGATES AND THE USE THEREOF AS VACCINES

Cross Reference to Related Application

This application is a continuation-in part of U.S. Application Serial No. 07/487,898 filed March 6, 1990, which is a continuation-in-part of U.S. Application Serial No. 07/385,860 filed July 27, 1989, the disclosures of which are fully incorporated by reference herein.

FIELD OF THE INVENTION:

This invention is in the field of pharmaceutical compositions and the use thereof as vaccines.

BACKGROUND OF THE INVENTION:

Each year approximately 194,000 patients in U.S. hospitals develop bacteremia; of these, about 75,000 die. Maki, D.G., in Nosocomial Infections, R.E. Dixon (ed.), pages 183-196, Yorke Medical Books, New York (1981). This high frequency of mortality occurs despite the aggressive use of potent antibiotics. The shortcomings of antibiotic therapy may be attributed to the relative impermeability of the outer membrane of Gram-negative bacteria to the drugs and to the inability of the drugs to counteract the lethal effects of bacterial endotoxin. Ziegler, E.J., et al., N. Engl. J. Med. 307:1225-1230 (1982); Bogard, W.C.Jr et.al., Infect. Immun. 55:899-908 (1987).

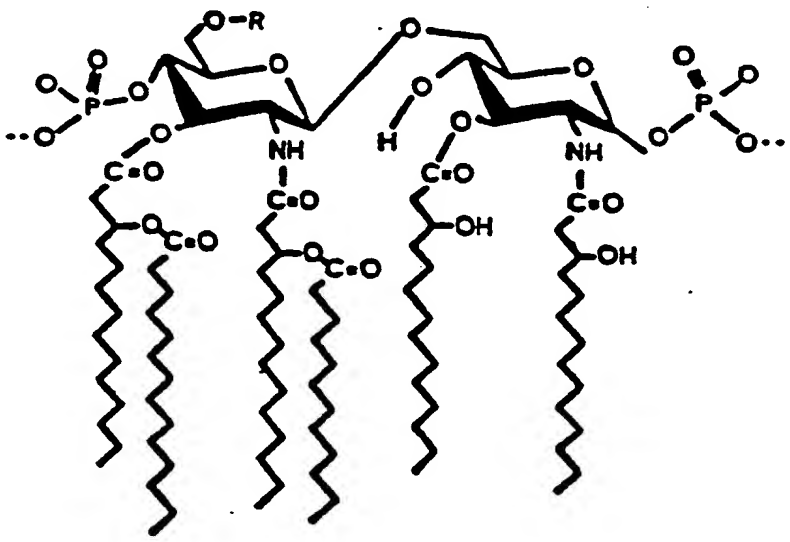
One approach toward counteracting the lethal effects of lipopolysaccharide (LPS) endotoxin is the administration of anti-LPS anti-

serum. Such antiserum may also facilitate the removal of Gram-negative bacteria by the reticuloendothelial system. Several workers have demonstrated successful broad-spectrum protection against challenge by several Gram-negative organisms or endotoxins in different animal models by using rough mutants of E. coli or S. minnesota as immunizing agents to generate antisera. Braude, A.I., et al., J. Immunol. 108:505-512 (1972); Braude, A.I., et al., J. Infect. Dis. 136:S167-S173 (1977); Dunn, D.L., et al., Surgery 92:212-219 (1982); McCabe, W.R., J. Immunol. 108:601-610 (1972); McCabe, W.R., et al., J. Infect. Dis. 136:S161-S166 (1977); and Ziegler, E.J., et al., J. Immunol. 111:433-438 (1973). These mutants lack the O-specific polysaccharide and express on their surface portions of the LPS core which presumably contains antigenic determinants shared by most Gram-negative organisms. Passive immunization of patients with human antisera raised against the rough mutant E. coli J5 has been shown to be an efficacious supplement to antibiotics for the control of nosocomial infections. Ziegler, E.J., et al., N. Engl. J. Med. 307:1225-1230 (1982).

It is now recognized that most of the biological activity of bacterial endotoxins reside in the lipid A moiety of the LPS molecule. Luderitz, O., et al., Curr. Top. Membr. Transp. 17:79-151 (1982). Typical Gram-negative bacterial LPS has three major structural regions: the O-polysaccharide, the R-core oligosaccharide, and lipid A. The structure of the O-polysaccharide is highly variable between organisms, even in the same species. Its antigenicity serves as a basis for serotyping the bacteria. The R region is a bridge between the O-antigen and lipid A; its structure is similar in most Gram-negative bacteria. Antibodies to LPS may promote phagocytosis or the death of the bacteria. The O-antigen is the most antigenic component of the LPS, yet it has little known toxicity. Lipid A, in contrast, contains the toxic center of the molecule and is remarkably similar in structure across a wide range of bacterial genera.

It is believed that the lipid A region of LPS is responsible for a complex array of inflammatory responses to tissue invasion by Gram-negative bacteria by directly stimulating host cells such as macrophages, neutrophils, and endothelial cells which mediate the inflammatory changes. Responses are both toxic (hypotension, coagulation disturbances, death) and beneficial to the infected host (enhancement of antibody formation, mobilization of phagocytes, acute phase protein synthesis).

The structure of lipid A is depicted as Formula (I) below:



(I)

Lipid A is a glucosamine disaccharide that is phosphorylated at positions 1 and 4′ and has six or seven esterified fatty acids. Four molecules of 3-hydroxytetradecanoate are attached to the glucosamine disaccharide at positions 2, 3, 2′, and 3′; the hydroxyl groups of the 3′-OH-14:0 residues at positions 2′ and 3′ (and sometimes 2) are substituted with normal fatty acids (dodecanoate, tetradecanoate, hexadecanoate) to form acyloxyacyl groups.

In order to gain insight into the structure-activity relationship of lipid A, the biological activity of chemically synthesized lipid A analogs and biosynthetic precursors of lipid A has been examined. For example, Luderitz, O., et al., Rev. Infect. Dis. 6:428-431 (1984),

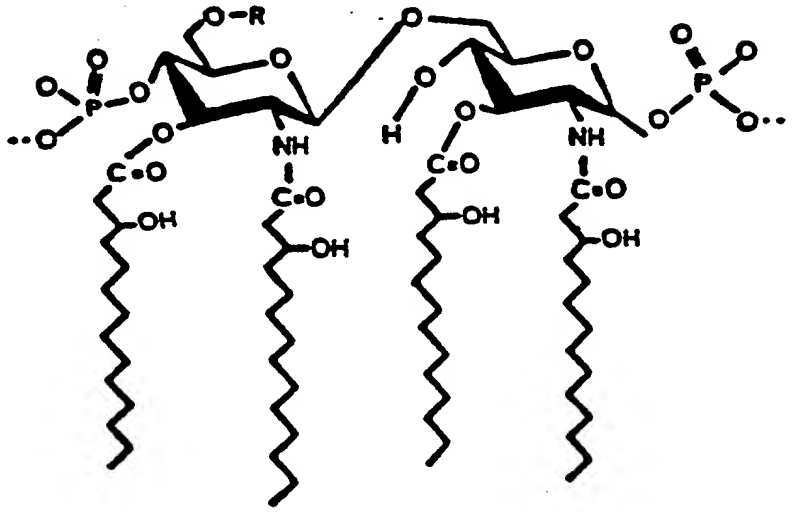
disclose the preparation of lipid A analogs and the relationship thereof between the chemical structure and biologic activity. The authors report that phosphate substitution of the glucosamine disaccharide is not essential for the expression of antigenicity, and that the amine-bound 3-hydroxyacyl residues can be replaced by non-hydroxylated fatty acids without reduction of activity. The authors also indicate that the immunodeterminant structure comprises the linkage region of the amide-linked fatty acids and glucosamine.

Galanos, C., et al., Rev. Infect. Dis. 6:546-552 (1984), disclose the preparation of semisynthetic lipid A immunogens comprising glucosamine disaccharide carrying one amide-linked 3-hydroxymyristic acid conjugated to a protein (edestin) for immunization of rabbits. Antibodies were produced in titers comparable to those obtained by immunization under similar conditions with acid-treated bacteria coated with lipid A.

Behling, U.H., et al., J. Immunol. 117:847-851 (1976), disclose the preparation of synthetic glycolipid adjuvants comprising N-palmitoyl-D-glucosamine, N-oleyl-D-glucosamine, N-myristoyl-D-glucosamine, N-decanoyl-D-glucosamine, and N-stearoyl-D-glucosamine. The authors report that comparable or superior enhancement of the immune response was obtained with the synthetic glycolipids in comparison to the use of LPS endotoxin in assays measuring anti-sheep red blood cell (SRBC) or gamma-globulin (HGG) hemagglutinin titers. The authors also report that the synthetic glycolipids have no activity in chick embryo lethality, Shwartzman skin assay, and Limulus lysate tests which are characteristic as well as sensitive assays of endotoxicity. Therefore, the authors conclude that the mitogenic and adjuvant effects by otherwise nonendotoxic glycolipids suggest that not all biologic properties of the endotoxin are related to one structural subunit or to one structural feature of this very complex molecule.

Hodgins, D.S., PCT Application Publication No. WO87/07297, published December 3, 1987, discloses LPS derivatives of reduced

toxicity obtained by treatment of lipid A with an acyloxyacyl hydrolase to give a compound of the Formula (II):



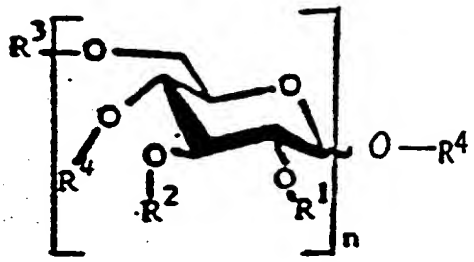
(II)

This altered bacterial LPS is therapeutically useful as a vaccine to prevent Gram-negative bacterial diseases by inducing antibodies to LPS, as an antidote to treat or prevent Gram-negative bacterial sepsis, or as an adjuvant to enhance formation of antibodies to other antigens. The acyloxyacyl hydrolase itself may also be prophylactically or therapeutically useful to detoxify endogenous LPS in patients with Gram-negative bacterial diseases. This enzyme may also be used to remove toxic LPS from therapeutic injectants.

Despite the development of lipid A analogs as immunogens for induction of active immunity to LPS, a need continues to exist for new compounds which can be used in the treatment or prevention of septic shock in animals.

SUMMARY OF THE INVENTION

The invention relates to a lipid A analog/immunogenic carrier conjugate, wherein said lipid A analog the following Formula (III):



(III)

wherein n is 1 or 2;

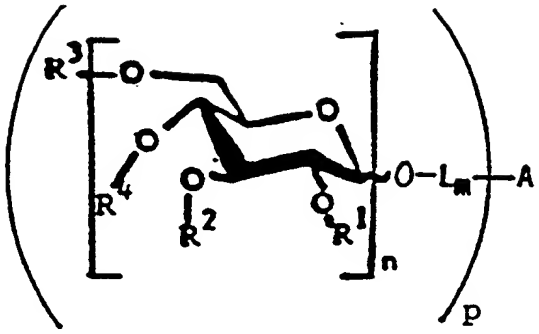
R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, a C_2-C_{18} acyl group, a 3-hydroxy C_3-C_{18} acyl groups, a 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl group and a linkage to an immunogenic carrier;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group, a phosphate group or a linkage to an immunogenic carrier;

with the proviso that one of R^1, R^3 or R^4 is a linkage to an immunogenic carrier, wherein said linkage does not interfere substantially with the ability of the lipid A analog to stimulate an immune response in an animal.

In particular, the invention relates to a lipid A analog/-immunogenic carrier conjugate having the following Formula (IV):



(IV)

wherein n, R^1, R^2, R^3 and R^4 are defined above;

A is an immunogenic carrier;

m is 0 or 1;

p is 1 to 200;

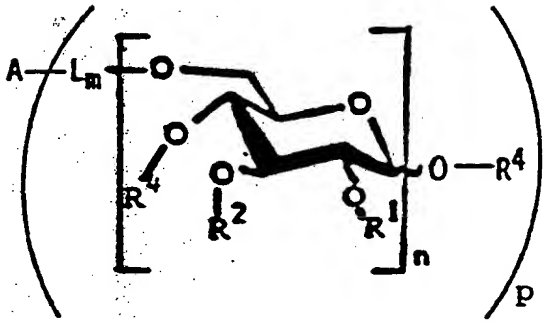
L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group.

The invention also relates to a lipid A analog/immunogenic carrier conjugate having the following Formula (V):



(V)

wherein n, m, p, L, R^1, R^2, R^3 and R^4 are defined above.

The invention also relates to a vaccine for the prophylaxis of septic shock comprising a lipid A analog/immunogenic carrier conjugate of the present invention together with a pharmaceutically acceptable carrier or adjuvant.

The invention also relates to a method for treating or preventing septic shock in an animal comprising administering a pharmaceutical composition to an animal comprising a lipid A analog/immunogenic carrier conjugate of the invention; and

a pharmaceutically acceptable carrier;

wherein said lipid A analog/immunogenic carrier conjugate is present in an amount effective to induce active immunity to LPS in an animal.

The invention also relates to intermediates useful for the preparation of the lipid A analog/immunogenic carrier conjugates of the invention.

Unexpectedly, the inventors of the present application have discovered that acyl derivatives of glucose and gentiobiose, when linked to an immunogenic carrier, induce active immunity to LPS in animals. This discovery allows the ready preparation of inexpensive vaccines which may be used for prophylaxis of septic shock.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates to lipid A analog/immunogenic carrier conjugates comprising glucose or a polyglucose molecule such as gentiobiose linked to an immunogenic carrier, wherein at least C^3 position of the glucose or gentiobiose moieties are substituted with an acyl group (R^2). The immunogenic carrier may be linked to any free hydroxyl group on the Lipid A analog molecule, so long as the conjugate induces active immunity to LPS when administered to an animal. Preferably, the immunogenic carrier is linked to the C^1 or C^6 position of the glucose or polyglucose moiety.

Equivalents of the lipid A analog/immunogenic carrier conjugates of the present invention include all non-amino saccharides and polysaccharides acylated at least at the C^3 position of at least one of the saccharide moieties and linked to an immunogenic carrier, wherein the conjugate induces active immunity to LPS when administered to an animal.

A lipid A analog/immunogenic carrier conjugate of the invention is considered to induce active immunity to LPS wherein when the conjugate is administered to an animal, anti-LPS antibodies are produced by the animal. The efficacy of the anti-LPS antibodies can be tested, for example, by administering the sera of an immunized animal to a second animal followed by challenge of the second animal with LPS-producing bacteria (see Example 7, below). Where administration of the anti-LPS sera results in enhanced survival of the second animal, the conjugate is considered to induce immunity to LPS.

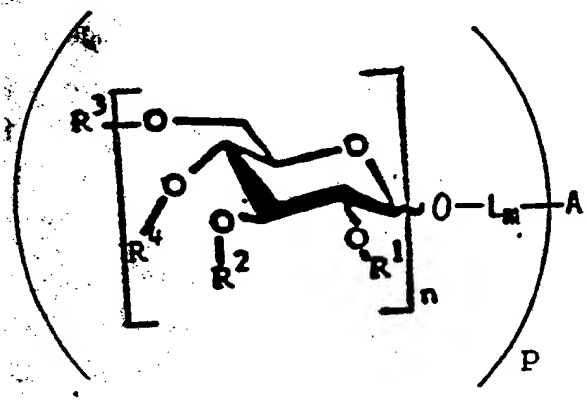
Anti-LPS antibodies can also be tested by inhibition of the Shwartzman reaction, a well-known biological response to LPS (Lee, L. et al., In: Zweifach, B.W. et al. (eds.) THE INFLAMMATORY PROCESS, Academic Press, NY, 1965, p. 791). Animals, particularly rabbits, actively immunized with LPS or with one of the compositions of the present invention are given an appropriate LPS challenge to induce a localized Shwartzman reaction. Prevention or amelioration of the erythema, hemorrhage or necrotic reaction is evidence for the presence

of anti-LPS antibodies. Alternatively, serum from an animal immunized with LPS or with one of the compositions of the present invention is transferred to a naive rabbit, which is then tested for a Shwartzman reaction. Inhibition of the reaction is a measure of antibody activity.

The vaccines of the present invention are useful for the prophylaxis of septic shock in an animal which is due to the release of LPS endotoxin by Gram negative microorganisms. Such Gram negative microorganisms include, but are not limited to Salmonella, Escherichia, Hemophilus and Neisseria, Klebsiella, Shigella, Pseudomonas, Enterobacter, Acinetobacter, and Bacteroides. See Bergey's Manual of Systematic Microbiology.

The conjugates of the invention are useful as vaccines which induce active immunity toward LPS in animals. Preferably, such animals are humans, however the invention is not intended to be so limiting. Any animal which may experience the beneficial effects of the vaccines of the invention are within the scope of animals which may be treated according to the claimed invention.

In particular, the invention relates to lipid A analog/immunogenic carrier conjugates having the Formula (IV):



(IV)

wherein A is an immunogenic carrier;

L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

m is 0 or 1;

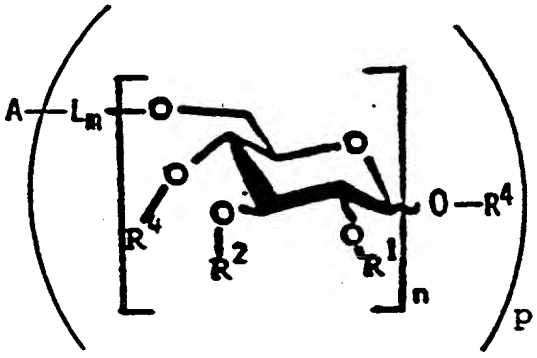
n is 1 or 2;

p is 1 to 200;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group.

The invention also relates to a lipid A analog/immunogenic carrier conjugate having the following Formula (V):



(V)

wherein n, m, p, L, R^1, R^2, R^3 and R^4 are defined above.

By the term "immunogenic carrier" is intended any macromolecule which is capable of inducing an immunogenic reaction in an animal. Since many small molecules such as the lipid A analogs of the invention do not induce active immunity by themselves, it is necessary to conjugate the analog to an immunogenic carrier to induce production of antibodies which are specific for the small molecule. Such immunogenic carriers include, but are not limited to, proteins such as

bovine serum albumin, diphtheria toxoid, tetanus toxoid, edestin, keyhole-limpet hemocyanin, Pseudomonal Toxin A, choleragenoid, cholera toxin, pertussis toxin, viral proteins, and eukaryotic proteins such as interferons, interleukins, or tumor necrosis factor. Such proteins may be obtained from natural or recombinant sources according to methods known to those skilled in the art. When obtained from recombinant sources, the immunogenic carrier may comprise a protein fragment comprising at least the immunogenic portion of the molecule. Other known immunogenic macromolecules which may be used in the practice of the invention include, but are not limited to, polysaccharides, tRNA, nonmetabolizable synthetic polymers such as polyvinylamine, polymethacrylic acid polyvinylpyrrolidone, mixed polycondensates (with relatively high molecular weight) of 4'4'-diaminodiphenylmethane-3,3'-dicarboxylic acid and 4-nitro-2-aminobenzoic acid (See Sela, M., Science 166:1365-1374 (1969)) or glycolipids, lipids or carbohydrates. Preferably, the immunogenic carrier is a protein.

By the term "linker group" is intended one or more bifunctional molecules which can be used to link the immunogenic carrier to the lipid A analog and which do not interfere with the production of anti-lipid A antibodies in vivo. The linker group may be attached to any part of the glucose or gentiobiose moiety so long as the point of attachment does not interfere with the production of anti-lipid A antibodies in vivo and thus interfere with the induction of active immunity.

Examples of linker groups which can be used to link the lipid A analog to the immunogenic carrier may comprise

$$-(CH_2)_q-NH-, \qquad \text{(VI)}$$

wherein q is 2-10;

$$-(CH_2)_q-NH-\overset{O}{\overset{\|}{C}}-(CH_2)_x-\overset{O}{\overset{\|}{C}}-, \qquad \text{(VII)}$$

wherein q = 2-5,

x = 2-12; and

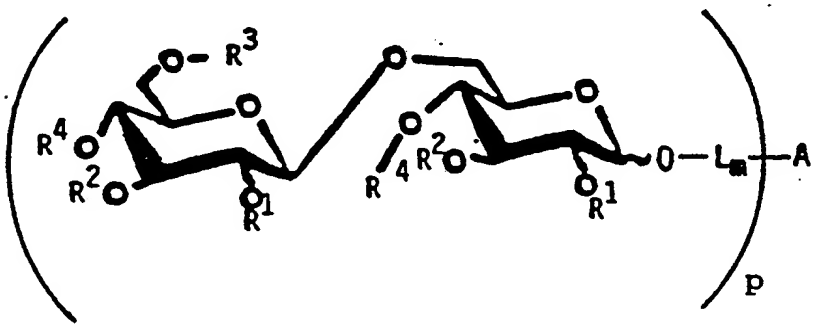
$$-(CH_2)_y-\overset{O}{\overset{\|}{C}}-, \qquad \text{(VIIII)}$$

wherein

y = 1-3.

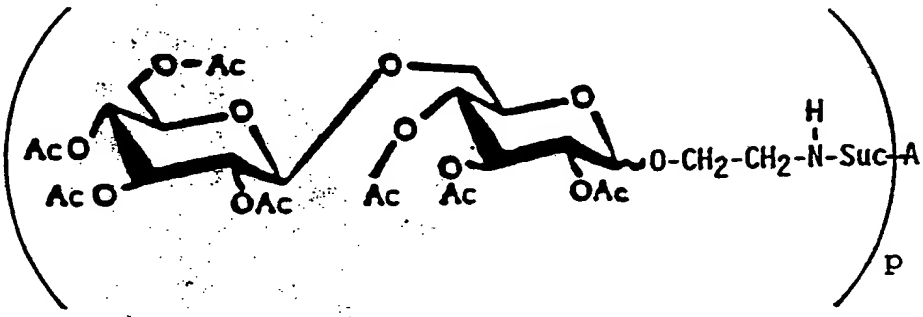
Typical acyl groups which can be substituted on the carbohydrate moiety include, but are not limited to, acetate, propionate, butanoate, pentanoate, hexanoate, heptanoate, octanoate, nonanoate, decanoate, palmitoyl, olelyl, myristoyl, stearoyl, 3-hydroxybutanoate, 3-hydroxypentanoate, 3-hydroxyhexanoate, 3-hydroxyheptanoate, 3-hydroxyoctanoate, 3-hydroxynonanoate, 3-hydroxydecanoate, 3-hydroxydecanoate, 3-hydroxypalmitoyl, 3-hydroxyoleyl, 3-hydroxymyristoyl, and 3-hydroxystearoyl groups. Also included within the scope of R groups include the 3-(C_2-C_{12} acyloxy)-substituted aforementioned C_3-C_{18} acyl groups wherein the C_2-C_{12} acyloxy groups include, but are not limited to, acetate, propanoate, butanoate, pentanoate, hexanoate, heptanoate, octanoate, nonanoate, decanoate, and dodecanoate groups.

Preferred lipid A analog/immunogenic carrier conjugates are derived from gentiobiose and have the following Formula (IX):



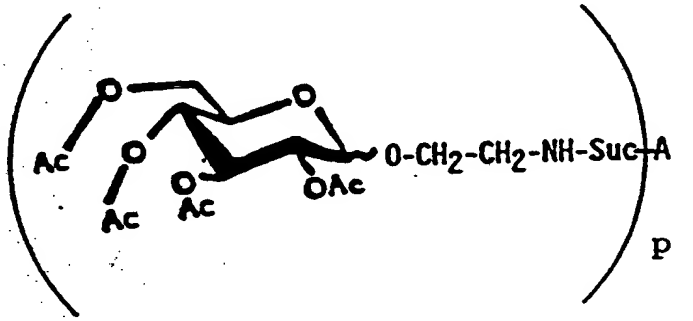
(IX)

A preferred lipid A analog/immunogenic carrier conjugate is gentiobiose peracetate linked at the C^1-position to an immunogenic carrier having the following Formula (X):



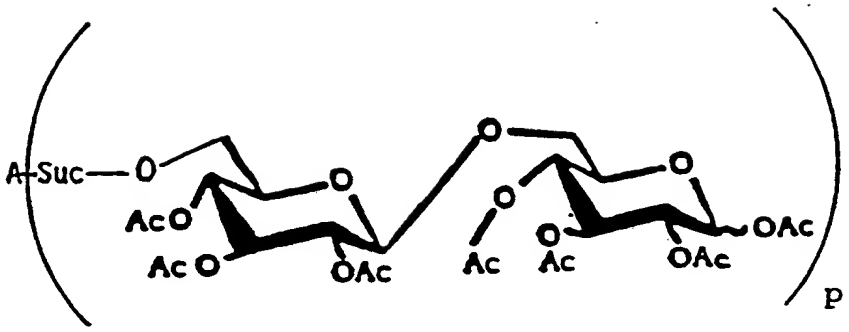
(X)

Another preferred lipid A analog/immunogenic carrier conjugate is an acyl glucose molecule linked at the C^1-position having the Formula (XI):



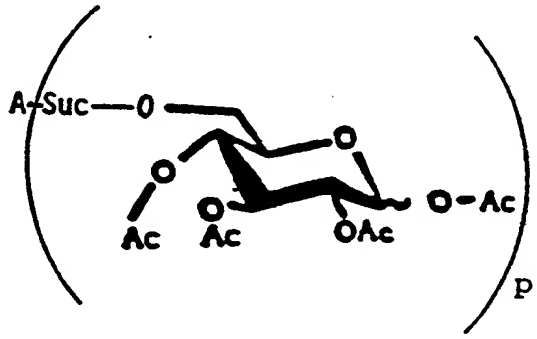
(XI)

Another preferred lipid A analog/immunogenic carrier conjugate is an acyl gentiobiose molecule linked at the $C^{6'}$-position to an immunogenic carrier having the Formula (XII):



(XII)

Another preferred lipid A analog/immunogenic carrier conjugate is an acyl glucose molecule linked at the $C^{6'}$-position to an immunogenic carrier having the Formula (XIII):



(XIII)

The lipid A analog/immunogenic carrier conjugates linked at the C^{1}-position may be prepared (see Scheme I) by treatment of an appropriately substituted and protected carbohydrate with a reagent capable of linking the carbohydrate to the immunogenic carrier having Formula (XIV) either directly or through a linker group. For example, gentiobiose or glucose peracetate (Formula (XV), n = 1 or 2; R^1, R^2, R^3 and R^4 = Ac) may be treated with HBr in glacial acetic acid to give the acetobromosaccharide derivative (XVI). The acetobromo derivative (XVI) may then be reacted with a linker such as 2-aminoethanol (XVII), 3-aminopropanol, 4-aminobutanol, or 5-aminopentanol, to give the aminoethyl peracetate saccharide derivative (XVIII) which may be

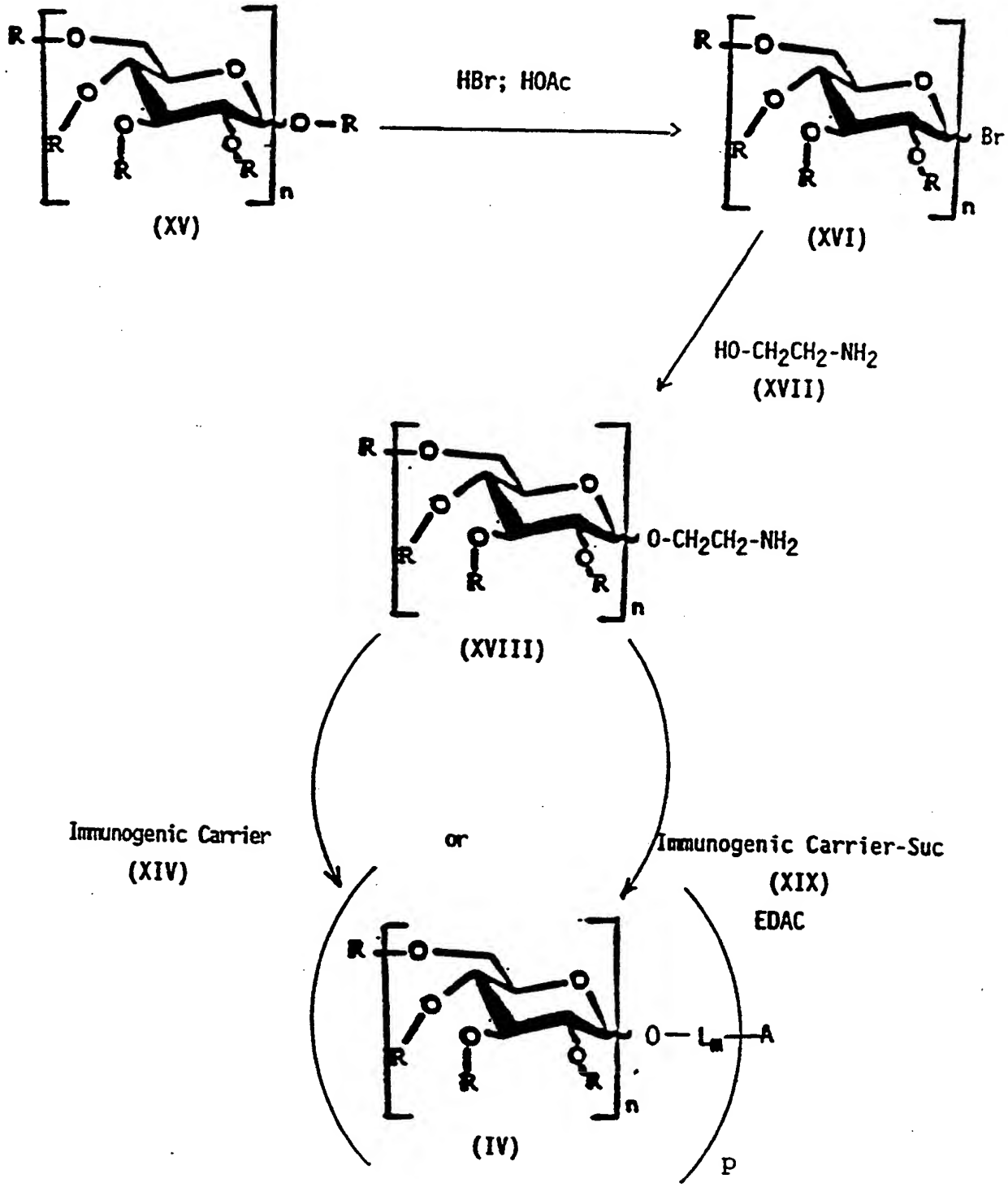
coupled to the immunogenic carrier (XIV) with, for example, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDAC) to give (IV). The use of EDAC to form conjugates between amino containing substances and proteins is disclosed in U.S. Patent No. 4,526,714 to Feijen et al.

Alternatively, the immunogenic peptide may be derivatized with a second bifunctional spacer group, such as succinic acid or any of the other linker groups having two carboxyl groups, to give the derivative (XIX). The derivative having Formula (XIX) may then be condensed with (XVIII) in the presence of EDAC to give (IV).

Where R^4 is a phosphate group, the hydroxy group may be phosphorylated prior to preparation of the conjugate by reacting an appropriately protected glucose or gentiobiose compound with the product of phosphorous acid and N-methylimidazole in the presence of $HgCl_2$. This reaction allows the preparation of the phosphate ester at the free hydroxyl group.

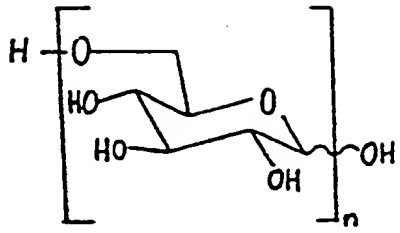
The ratio of lipid A analog molecules per immunogenic carrier molecule may vary considerably according to the molecular weight of the immunogenic carrier, the number of binding sites on the immunogenic carrier capable of being coupled to the lipid A molecule, and the antigenic characteristics of the particular lipid A molecule. In general, the ratio of lipid A analog molecules to immunogenic carrier molecules may be about 1:1 to about 200:1. Preferably, the ratio may range from about 5:1 to about 100:1. More preferably, where the immunogenic carrier is diphtheria toxoid, the ratio of lipid A analog molecules to diphtheria toxoid molecules may range from about 5:1 to about 40:1.

Scheme I


HBr; HOAc
(XV)
(XVI)
HO-CH2CH2-NH2
(XVII)
O-CH2CH2-NH2
(XVIII)
Immunogenic Carrier
(XIV)
or
Immunogenic Carrier-Suc
(XIX)
EDAC
(IV)

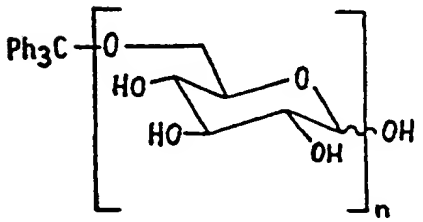
Where the immunogenic carrier is linked to the lipid A molecule at the C^6-position, the conjugates may be prepared according to scheme II by the selective tritylation of, for example, the $C^{6'}$-position of gentiobiose (XX) with trityl chloride (XXI) to give the $C^{6'}$-trityl gentiobiose derivative (XXII). Acylation of the remaining hydroxyl groups with, for example, acetic anhydride gives 6'-O-tritylgentiobiose heptaacetate (XXIII). Removal of the trityl group with, for example, HBr gives the $C^{6'}$-hydroxygentiobiose heptaacetate derivative (XXIV) which may be linked to an immunogenic carrier such as the succinate derivative of diphtheria toxoid to give (XXV).

Scheme II



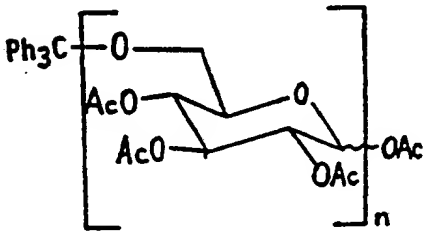
(XX)

Ph_3C-Cl (XXI), pyr.



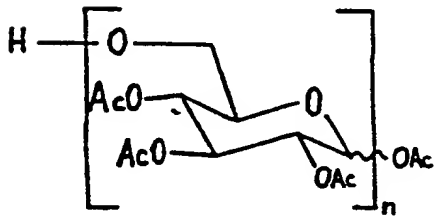
(XXII)

Ac_2O, pry.



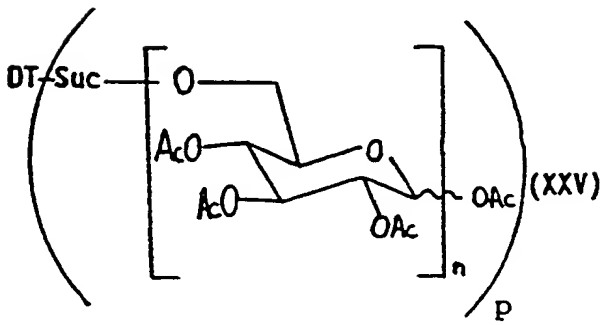
(XXIII)

HBr, AcOH



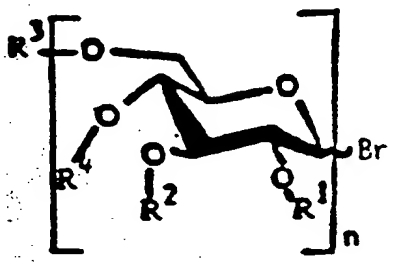
(XXIV)

DT-Suc; EDAC



(XXV)

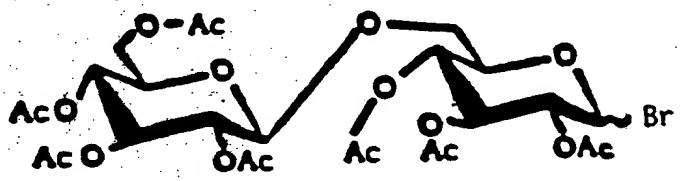
The invention also relates to intermediates useful for the preparation of the lipid A analog/immunogenic carriers of the invention. In particular, the invention relates to a 1-bromo-saccharide having the Formula (XXVI):



(XXVI)

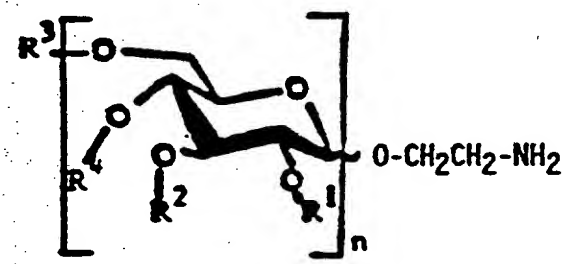
wherein n=2.

The invention also relates to the intermediate having Formula (XXVII):



(XXVII)

The invention also relates to lipid A analog/linker conjugates having the Formula (XXVIII):

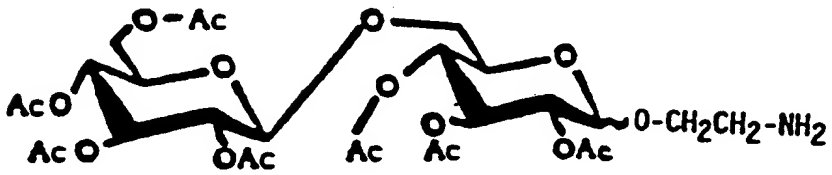


(XXVIII)

In particular, the invention relates to a lipid A analog/linker conjugate having the Formula (XXIX):

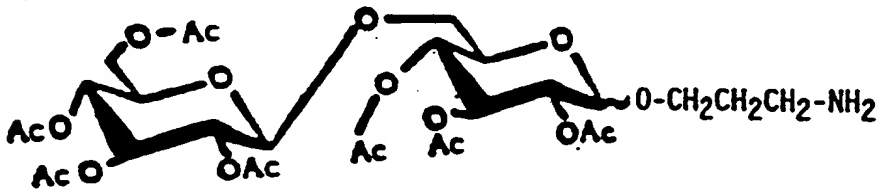
(XXIX)

The invention also relates to a lipid A analog/linker conjugate having the Formula (XXX):



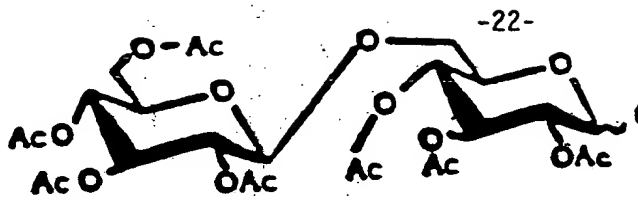
(XXX)

The invention also relates to a lipid A analog/linker conjugate having the Formula (XXXI):



(XXXI)

The invention also relates to a lipid A analog/linker conjugate having the Formula (XXXII):



(XXIII)

The conjugates of the invention may be purified by any method known to those of ordinary skill in the art. For example, the conjugates may be purified by reverse phase chromatography, ion exchange chromatography, size exclusion chromatography, or by dialyzing the reaction product against water followed by freeze-drying. Alternatively, the conjugates may be purified by passing a solution of the conjugate through a column having anti-lipid A antibodies immobilized on a solid phase support. (See the Examples section of the application for a method of preparing antibodies to lipid A.)

Administration of the vaccine comprising the lipid A analog/immunogenic carrier conjugate of the present invention may be parenteral, intravenous, intramuscular, subcutaneous, intranasal, or any other suitable means. Preferably, administration is by subcutaneous or intramuscular means. The dosage administered may be dependent upon the age, weight, kind of concurrent treatment, if any, and nature of the antigen administered. In general, the conjugate may be administered at a dosage of 0.001 to 25.0 μg/kg of animal weight. The initial dose may be followed up with a booster dosage after a period of 4 weeks to enhance the immunogenic response. Further booster doses every six months may be administered for as long as the risk of infection and septic shock exists.

The lipid A analog/immunogenic carrier conjugates useful in the method of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, or phosphate-buffered saline, or any such carrier in which the compounds used in the method of the present invention have suitable solubility proper-

ties for use in the method of the present invention. The vaccines may be in the form of single dose preparations or in multi-dose flasks which can be used for mass vaccination programs. Reference is made to Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, Osol (ed.) (1980); and New Trends and Developments in Vaccines, Voller et al. (eds.), University Park Press, Baltimore, MD (1978), for methods of preparing and using vaccines.

The vaccines of the invention comprising the lipid A analog/-immunogenic carrier conjugates of the invention may further comprise adjuvants which enhance production of lipid A analog-specific antibodies. Such adjuvants include, but are not limited to, various oil formulations such as Freund's complete adjuvant, the dipeptide known as MDP, saponin, aluminum hydroxide, Bordetella pertussis, dephosphorylated lipid A, or an interferon.

Freund's adjuvant is an emulsion of mineral oil and water which is mixed with the immunogenic substance. Although Freund's adjuvant is powerful, it is usually not administered to humans. Instead, the adjuvant alum (aluminum hydroxide) may be used for administration to a human. The conjugate may be absorbed onto the aluminum hydroxide from which it is slowly released after injection.

The lipid A analog/immunogenic peptide conjugate may also be encapsulated within liposomes according to Fullerton, U.S. Patent No. 4,235,877.

Having now generally described the invention, the same may be further understood by reference to the following examples, which are not intended to be limiting unless so expressly stated.

EXAMPLES

Example 1: Preparation of a Gentiobiose peracetate-BSA conjugate

Gentiobiose octaacetate, obtained from Sigma Chemical Co. (St. Louis, MO), was converted into the acetobromo derivative by dissolution in dichloromethane (1 gm in 5 ml) and treatment with 33% HBr in glacial acetic acid (10 ml) (Fluka) at 5°C for 45 minutes. A volume of dichloromethane equal to the total volume of the reaction mixture was added and the mixture transferred to a separatory funnel. The organic phase was washed with an equal volume of saturated sodium bicarbonate solution (3-4 times), followed by two portions of water. The organic layer was then dried over $MgSO_4$, filtered, and the solvent evaporated to give a syrup. The acetobromogentiobiose was crystallized from dichloromethane and diethyl ether, filtered and dried in a vacuum desiccator. The crystalline compound gave a single spot on T.L.C. and the H-1 NMR spectrum was correct.

Acetobromogentiobiose (5 gm in 5 ml chloroform) was coupled to 2-aminoethanol (0.5 ml, approx. 0.5 gm, in 1 ml chloroform) in the presence of drierite (1 gm; W.A. Hammond Drierite Co.). The mixture was stirred overnight at room temperature then filtered. The resulting aminoethyl gentiobiose heptaacetate (AGH) was first purified by chromatography on a column of silica gel (1.6X24 cm, 50 ml total volume) using an ethyl acetate-ethanol solvent mixture (19:1) as the eluting solvent. The fractions were screened by TLC using ethyl acetate-ethanol (19:1) to elute. The fractions containing the aminoethyl derivative were combined, the solvent evaporated in vacuo, and the product crystallized from hot ethanol at 55°C. The crystalline compound gave a single spot on T.L.C. and had a melting point of 176-177°C. Elemental analysis showed C, 49.44%; H, 6.05%; N, 2.1% (calculated for $C_{28}H_{40}O_{28}$ N: C, 49.56%; H, 5.94%; N, 2.06%).

In order to increase the number of free carboxyl groups and solubility of bovine serum albumin (BSA), the BSA (10-50 mg in PBS at 4-5 mg/ml) was slowly treated with succinic anhydride (5-10 mg/mg protein; Aldrich Gold Label) at pH 8-9. The pH was maintained at 8-8.5 by the addition of concentrated NaOH. The reaction mixture was then extensively dialyzed against water and the resulting succinylated BSA (Suc-BSA) freeze-dried.

Dioxane was the solvent found capable of dissolving both the protein carriers and the AGH ligand. Suc-BSA (10 mg) was coupled to AGH in 20% 1,4-dioxane (2-3 ml; Fisher Scientific) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (10 mg; EDAC). The pH was monitored using pH paper and maintained at 5-6 by the addition of dilute HCl. Three additional portions of EDAC (10 mg) were added after 30 minute intervals. The resulting conjugate (Suc-BSA-AGH) was dialyzed against water and then frozen. Analysis for reducing sugar by the phenol-sulfuric acid assay showed that the conjugate consisted of 27.4% AGH. This is about 35 moles of ligand/mole of protein.

Example 2: Preparation of a Gentiobiose Peracetate-DT Conjugate

Diphtheria Toxoid (DT) from SSVI (Bern, Switzerland) was coupled to AGH (obtained according to Example 1) in 25% 1,4-dioxane and 0.05M NaCl in the presence of EDAC. The resulting conjugate (DT-AGH) was dialyzed against water and freeze-dried. Analysis demonstrated that 16% of the conjugate consisted of AGH.

Example 3: Preparation of Aminopropylgentiobiose Heptaacetate-Succinyl-DT Conjugate

3-Aminopropanol was linked to acetobromogentiobiose (as described for 2-aminoethanol in Example 1). Diphtheria toxoid (DT) was succinylated (as described for BSA in Example 1) to give Suc-DT which was then coupled to aminopropylgentiobiose heptaacetate (APGH; according to Example 2) to give Suc-DT-APGH.

Example 4: Binding of Gentiobiose-Protein Carrier Conjugates to LPS specific antibodies

Although gentiobiose octaacetate is insoluble in water, the protein conjugates dissolve easily. These soluble conjugates were tested for their ability to bind anti-lipid A human monoclonal antibody (obtained by fusion of the heteromyeloma SHMA6(H4) with Epstein Barr virus (EBV) transformed lymphocytes from the spleen of a patient immunized with the J5 mutant of E. coli O111-B_4 which is deficient in the enzyme uridine 5'-diphosphategalactose 4-epimerase which prevents attachment of the side chains responsible for the marked antigenic diversity among Gram-negative bacteria) by enzyme-linked solid phase immunoassay (ELISA). As shown in Table 1, both BSA-gentiobiose octaacetate and diphtheria toxoid-gentiobiose octaacetate conjugates were able to bind the human monoclonal antibody. These experiments demonstrate that the important binding epitope remained intact during the conjugation process. Therefore, it would be expected by one of ordinary skill in the art that anti-lipid A antibodies would be produced in vivo upon administration of the lipid A analog/immunogenic carrier conjugates to an animal.

Table 1

Binding of the lipid A Antibody to
Gentiobiose Octaacetate/Protein Conjugate

Suc-BSA-AGH conjugate	2,560
Suc-BSA-Control	640
DT-AGH conjugate	48
DT control	0
AGH in ethanol	1,280
E. coli Lipid A	2,560
Suc-DT-APGH	3,200
Suc-DT (control)	0

Example 5 In Vivo Immunogenicity of the Gentiobiose Octaacetate/ Protein Conjugate in Rabbits

The gentiobiose octaacetate protein/diphtheria toxoid conjugate was administered to rabbits according to the immunization schedule outlined in Table 2. Antibody titers were then determined by ELISA. Sera from rabbits 1 and 2 was then assayed for antibodies specific for a series of antigens. Table 3 shows that the rabbit sera has reactivity with LPS and diphtheria toxin, but little reactivity with APGH.

Table 2

Rabbit No.	Vaccine	Dose	Adjuvant	Date
1 & 2	Suc-DT-APGH	200 ug	Freund's	4/11/88
	Lot AKB III-A	200 ug	Freund's	4/20/88
	Substitution: 5.2%[a]	200 ug	None	4/29/88
		150 ug	None	5/25/88
		150 ug	None	6/30/88
		150 ug	None	8/15/88
		150 ug	None	10/28/88
		150 ug	None	1/4/89
				Animals bled out 1/12/89
3 & 4	Suc-DT-APGH Lot AKB III-A-171 Substitution: 20%[a]			
5 & 6	Suc-DT-APGH Lot AKB V-33A Substitution: 4.4%[a]			
All four animals immunized		100 ug	Alum	11/2/88
on same schedule:		100 ug	Alum	11/30/88
		100 ug	None	12/31/88
		100 ug	None	1/13/89
		100 ug	None	2/17/89

[a]Substitution of succinylated diphtheria toxin by APGH expressed as weight percent.

Table 3

Antigen	Rabbit	Pre-bleed 4/11	Post bleeds 4/29	5/13	6/17	7/11
J5 LPS	1	150[a]	300	400	800	800
	2	200	200	300	600	1,000
Men Gr B LPS	1	100	200	300	200	300
	2	100	150	250	250	300
APGH	1	<100	<100	100	150	150
	2	<100	100	100	150	250
DT	1	300	1,600	6,400	12,800	>12,800
	2	300	12,800	12,800	>12,800	>12,800

[a]All data is expressed as the dilution giving an optical density reading of 0.4 units.

Rabbit sera was also assayed against a tetanus toxoid-APGH conjugate (TT-APGH), which does not have a spacer group. The results listed in Table 4 show that the antibodies are also specific for this conjugate, confirming that the antibodies are reactive with the APGH part of the molecule.

Table 4

Antigen 1/24/89	Rabbit	Pre-bleed	Post bleeds 1/4/89	1/12/89
TT-APGH (no spacer)				
	1	100[a]	12,800	
	2	100	>12,800	
	3	100	4,800	1,600
	4	100	800	1,600
	5	100	1,200	3,200
	6	100	600	800

[a]All data is expressed as the dilution giving an optical-density reading of 0.4 units.

Antibody specificity for LPS on boiled J5 was then determined by specific adsorption experiments. In each experiment, the sera of rabbits was diluted 1:500 and adsorbed twice with boiled J5 organisms at 10% volume of packed cells. The first adsorption was for 2 hours followed by a second overnight adsorption (both at 4°C). The J5 organisms were analyzed by GLC and found to be free of galactose. The sera from the final bleeds of rabbits 1 and 5 were then assayed pre- and post-adsorption by ELISA using TT-APGH. The results are shown in Table 5.

Table 5

Rabbit Number	% Reduction
#5	
Exp 1	39%
Exp 2	42%
#1	27%

[a]Percent reduction at O.D. = 0.4.

As shown in Table 5, there was significant reduction of antibody titer when the sera was adsorbed to J5 LPS. These data confirm the expectation that the sera of animals immunized with the conjugate is reactive with LPS on boiled J5.

Example 6 Preparation of 6'-Substituted Gentiobiose Heptaacetate Succinyl-Diphtheria Toxoid (SUC-DT) Conjugate

A. Preparation of 6'-O-Trityl-Gentiobiose

Gentiobiose (4 gm, Sigma) was added slowly with stirring to pyridine (60 ml) and to the resulting suspension was added with stirring triphenylmethylchloride (4 gm, Aldrich). The mixture was stirred overnight at room temperature.

The solvent was evaporated off in a rotary evaporator and the resident pyridine was removed by co-distillation with toluene (3 x 15 ml). The solid product was dissolved into dichloromethane (30 ml) and the solution was loaded onto a washed silica gel column (2.0 x 70 cm). The excess trityl chloride was first eluted with ethyl acetate. The 6'-O-trityl-gentiobiose was then eluted with methanol. The fractions containing the product were combined and evaporated to dryness.

B. Preparation of 6'-O-Trityl-Gentiobiose Heptaacetate

6'-O-Trityl-gentiobiose was dissolved into pyridine (35 ml) and to this solution was added with stirring 17 ml of acetic anhydride (Aldrich Chemical Corporation, Milwaukee, WI). The mixture was stirred overnight at room temperature and then poured into 400 ml of crushed ice. The resulting precipitate was filtered, washed with cold water and dried under vacuum (yield 5.5 gms).

C. Preparation of 6'-O-Hydroxy-Gentiobiose Heptaacetate (C-6'-HGH)

1.0 gm of 6'-O-trityl-gentiobiose heptaacetate was dissolved into 5.0 ml of glacial acetic acid and to this solution was added 33% HBr in glacial acetic acid (Fluka) to make 2% HBr. The mixture was poured into 50 ml of iced water and the resulting precipitate (trityl bromide) was filtered out.

The aqueous solution was extracted with dichloromethane (50 ml) and the organic phase was washed with saturated $NaHCO_3$ solution, cold water, dried with anhydrous sodium sulfate and evaporated to dryness. The solid product was crystallized from diethyl ether. Yield 150 mg.

D. Preparation of 6'-Hydroxy-Gentiobiose Heptaacetate Succinyl-Diphtheria Toxoid (SUC-DT) Conjugate.

Succinyl-DT (7.2 mg) was coupled to 6'-hydroxy-gentiobiose heptaacetate (3.5 mg) in 20% 1,4-dioxane (Fisher Scientific Co.) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (18 mg, EDAC). The pH was maintained between 5.7 and 6.0 with 0.1N HCl. Two additional portions of EDAC (10 mg) were added at 30 minute intervals. The resulting conjugate (6'-O-SucDT-GH) was dialyzed against water and then freeze dried. Analysis for reducing sugar by the phenol-sulfuric acid assay showed that the conjugate consisted of 20% 6'-HGH. This is about 30 moles of ligand/mole of protein.

Example 7 Immunogenicity Studies of 6'-O-SucDT-GH Vaccine

The vaccine was prepared for immunization studies by dissolving the lyophilized conjugate in sterile phosphate buffered saline to a final concentration of 200 ug/ml, followed by adsorption to aluminum oxide (Rhesorptar) overnight at 4°C. The vaccine was administered intramuscularly to NZW rabbits at 50 ug per dose, and sera were collected periodically during the immunization schedule. Vaccine was administered at weeks 0, 3, 6, 9, 12, and 16. Immunogenicity was

determined by ELISA using a heterologous tetanus conjugate vaccine (1'-O-SucDT-GH) as the plate antigen at a concentration of 2 ug/ml. Sera were run in triplicate beginning at a 1/50 dilution, and data reduced to optical density (O.D.) units, the titer at which the O.D. is equal to 1.0. The results for serial bleeds obtained 1 week after each dose shown in Table 6.

Table 6

Immunogenicity of 6'-O-Suc-DT-GH Vaccine

Rabbit #	Wk 0	Wk 1	Wk 4	Wk 7	Wk 10	Wk 13	Wk 17
R008	22	111	2,208	1,844	2,947	3,661	1,206
R009	20	27	443	1,053	2,423	13,097	6,884
R010	23	82	215	926	3,890	3,925	2,775
R012	15	55	474	663	2,597	2,897	2,080
R014	13	43	2,374	1,232	1,232	1,312	590

The results show that the vaccine induced ligand-specific antibodies in each rabbit immunized. To determine the protective efficacy of these antibodies, their ability to protect mice in a hog mucin challenge model was assayed. Pooled sera obtained at week 14 from immunized rabbits, or pooled sera obtained from control rabbits not receiving the vaccine, were sterile filtered and administered intraperitoneally to NSA mice at a dose of 1.0 ml per mouse. Two hours later the mice were challenged with from 4.8×10^3 to 4.8×10^5 *aeruginosa* suspended in a 14% preparation of hog mucin. Mice were examined hourly for the next 72 hours and the time of death recorded. Table 7 reports the results of these studies.

Table 7

Hog Mucin Challenge Model

Protection from Challenge with *Pseudomonas aeruginosa*

Group (n=10)	Challenge (cfu)	72 hr survival (%)	p*	Hours surviving (Mean ± S.E.M.)	p**
Immune	$4.8x10^3$	70	.0049	60.6±6.41	.0001
Normal	$4.8x10^3$	0		18.4±0.30	
Immune	$4.8x10^4$	40	.01	47.6±7.01	.0022
Normal	$4.8x10^4$	0		18.0±0	
Immune	$4.8x10^5$	30	.01	37.8±7.53	.0001
Normal	$4.8x10^5$	0		18.0±0	

* X^2 analysis of immune group vs all controls receiving ≤ that challenge dose.

** Student's t test, one tailed

These data demonstrate that the vaccine was immunogenic and produced antibodies that protect mice against challenge with a virulent gram negative bacteria.

Example 8

Active and Passive Protection from a Localized Shwartzman Reaction

A. Active Immunization: Three rabbits were immunized twice with Suc-DT-APGH (see Example 3) in aluminum hydroxide gel (20:1, w/w) and subsequently received 4 injections of the antigen in saline. To test development of a localized Shwartzman reaction, rabbits each received an intracutaneous injection of 50 μg E. coli 06 LPS followed 23 hours later with 25 μg LPS in 0.1 ml saline, intravenously. Only 1 of 3 immunized rabbits showed a positive erythema, although no hemorrhagic reaction, whereas 3 of 3 non-immune rabbits were positive for erythema and 2 had hemorrhagic reactions. No necrosis was observed.

B. Passive Immunization: Rabbits whose sera were to be tested were immunized once with Suc-DT-APGH (Example 3) in complete Freund's adjuvant, followed by 7 injections of the antigen in saline. Serum obtained from these hyperimmunized rabbits had an antibody titer of 1:6400 measured in an ELISA with TT-APTGH.

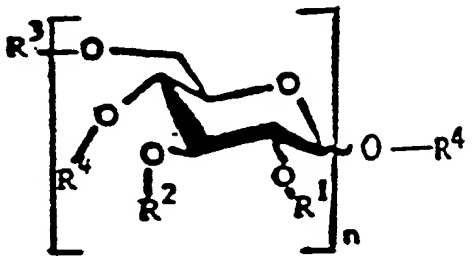
Test rabbits were injected intracutaneously with 100 μg E. coli 06 LPS in 0.2ml saline. After 21 hours, rabbits received 15 ml of the hyperimmune serum or normal serum intravenously (in the ear vein) and 2 hours later were challenged by intravenous injection of 20 μg LPS in 0.2 ml. In the control rabbits receiving normal serum, 4 of 5 showed hemorrhage and 1 had tissue necrosis. Only 1 of 5 rabbits receiving hyperimmune serum showed a positive hemorrhagic reaction and none had any signs of necrosis.

Therefore both active and passive immunization against Suc-DT-APGH afforded significant protection against the LPS-induced Shwartzman reaction, further confirming the capacity of the composition to induce anti-LPS antibodies capable of acting in vivo.

Having now fully described this invention, it will be understood by those of skill in the art that the same can be performed with any wide equivalent range of compositions, concentrations, formulation and other parameters without affecting the scope of the invention or any embodiment.

WHAT IS CLAIMED IS:

1. A lipid A analog/immunogenic carrier conjugate comprising a lipid A analog of the formula

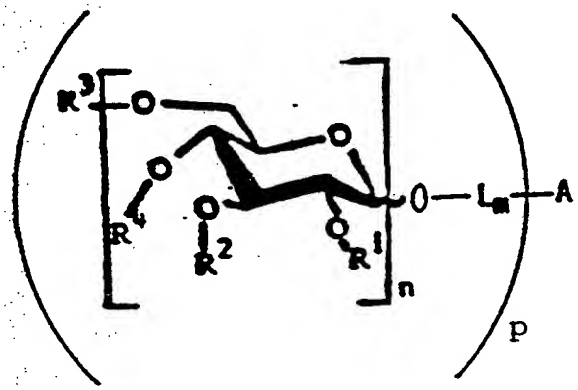


wherein R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, a 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl group or a linkage to an immunogenic carrier;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group, a phosphate group or a linkage to an immunogenic carrier; n = 1 or 2;
with the proviso that one of R^1, R^3 or R^4 is a linkage to an immunogenic carrier, wherein said linkage does not interfere substantially with the ability of the lipid A analog to stimulate an immune response in an animal.

2. A lipid A analog/immunogenic carrier conjugate having the following formula:



wherein A is an immunogenic carrier;

m is 0 or 1;

n is 1 or 2;

p is 1 to 200;

L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group;

wherein said conjugate induces active immunity to LPS when administered to an animal.

3. The lipid A analog/immunogenic carrier conjugate of claim 2, wherein said linker group comprises -O-$(CH_2)_q$-NH-, wherein q is 2-3.

4. The lipid A analog/immunogenic carrier conjugate of claim 2, wherein L is

$$-(CH_2)_q\text{-NH-}\overset{O}{\overset{\|}{C}}\text{-}(CH_2)_x\text{-}\overset{O}{\overset{\|}{C}}\text{-},$$ wherein

q = 2-5, and

x = 2-12.

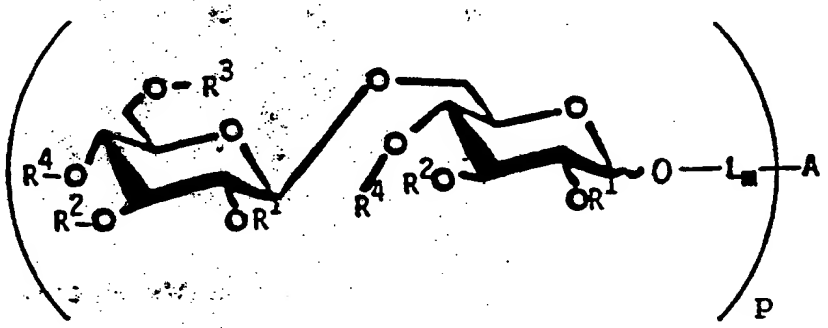
5. The lipid A analog/immunogenic carrier conjugate of claim 2, wherein L is

$-(CH_2)_y-\overset{O}{\overset{\|}{C}}-$, wherein

y = 1-3.

6. The lipid A analog/immunogenic carrier conjugate of claim 1, wherein said immunogenic carrier is a protein selected from the group consisting of bovine serum albumin, diphtheria toxoid, edestin, Toxin A and choleragenoid.

7. A lipid A analog/immunogenic carrier conjugate having the formula:



wherein A is an immunogenic carrier;

m is 0 or 1;

p is 1 to 200;

L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

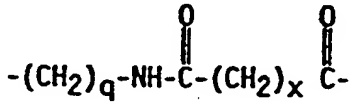
R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group;

wherein said conjugate induces active immunity to LPS when administered to an animal.

8. The conjugate of claim 7, wherein the immunogenic carrier is a protein selected from the group consisting of bovine serum albumin, diphtheria toxoid, edestin, Toxin A and choleragenoid.

9. The lipid A analog/immunogenic carrier conjugate of claim 7, wherein said linker group comprises -O-$(CH_2)_q$-NH-, wherein q is 2-5.

10. The lipid A analog/immunogenic carrier conjugate of claim 7, wherein said linker group comprises

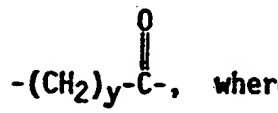


-$(CH_2)_q$-NH-C-$(CH_2)_x$ C-, wherein

q = 2-5, and

x = 2-12.

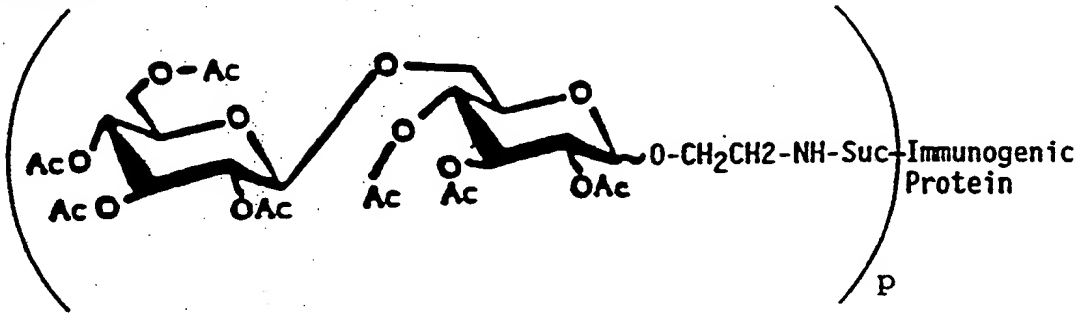
11. The lipid A analog/immunogenic carrier conjugate of claim 7, wherein L is



-$(CH_2)_y$-C-, wherein

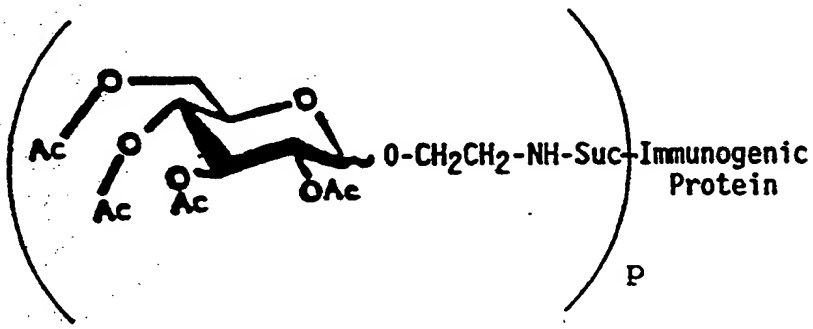
y = 1-3.

12. A lipid A analog/immunogenic carrier conjugate having the formula:



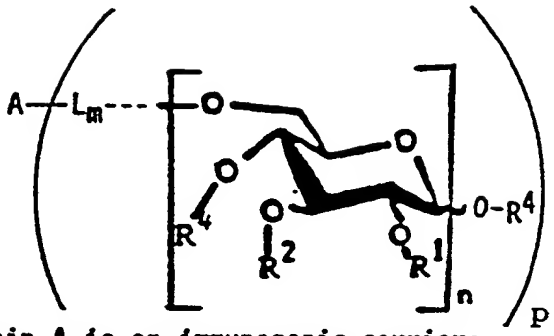
wherein p is 1-200.

13. A lipid A analog/immunogenic carrier conjugate having the formula:



wherein p is 1 to 200.

14. A lipid A analog/immunogenic carrier conjugate having the following formula:



wherein A is an immunogenic carrier;

m is 0 or 1;

n is 1 or 2;

p is 1 to 200;

L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group;

wherein said conjugate induces active immunity to LPS when administered to an animal.

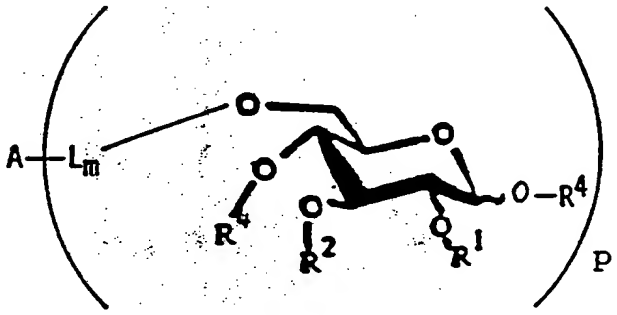
15. The lipid A analog/immunogenic carrier conjugate of claim 14, wherein L is

-C-$(CH_2)_x$-C-, wherein x = 2-12.

16. The lipid A analog/immunogenic carrier conjugate of claim 14, wherein said immunogenic carrier is a protein selected from the

group consisting of bovine serum albumin, diphtheria toxoid, edestin, Toxin A and choleragenoid.

17. A lipid A analog/immunogenic carrier conjugate having the formula:



wherein A is an immunogenic carrier;

m is 0 or 1;

p is 1 to 200;

L is a linker group which does not interfere substantially with the characteristic ability of the lipid A analog to stimulate an immune response in an animal;

R^1 and R^3 are selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

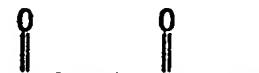
R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group;

wherein said conjugate induces active immunity to LPS when administered to an animal.

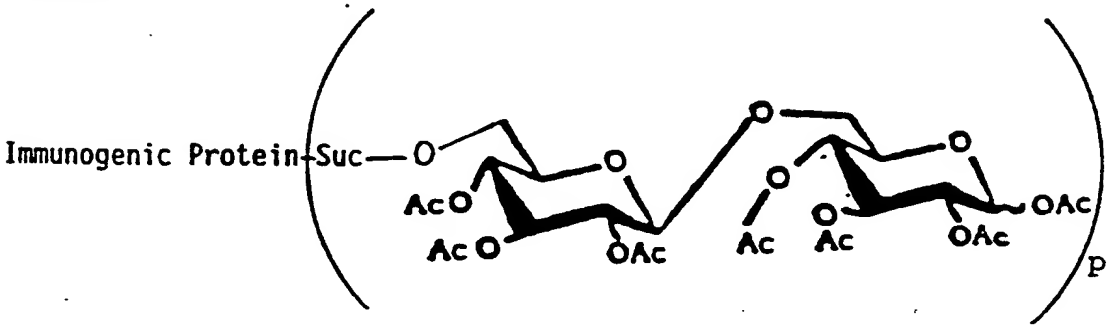
18. The conjugate of claim 17, wherein the immunogenic carrier is a protein selected from the group consisting of bovine serum albumin, diphtheria toxoid, edestin, Toxin A and choleragenoid.

19. The lipid A analog/immunogenic carrier conjugate of claim 17, wherein said linker group comprises



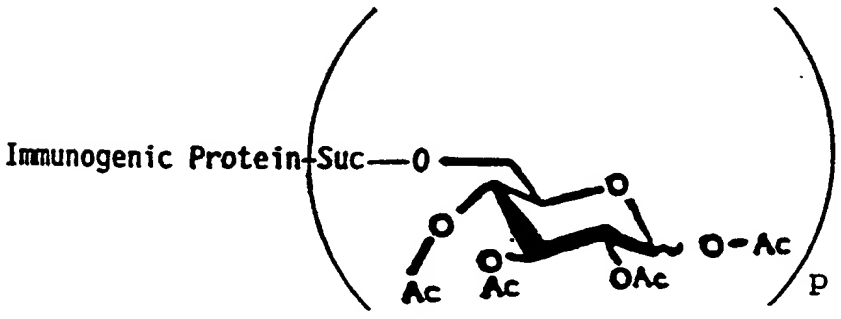
$-C-(CH_2)_x-C-$, wherein x = 2-12.

20. A lipid A analog/immunogenic carrier conjugate having the formula:



wherein p is 1-200.

21. A lipid A analog/immunogenic carrier conjugate having the formula:



wherein p is 1 to 200.

22. A vaccine for the prophylaxis of septic shock in an animal comprising:

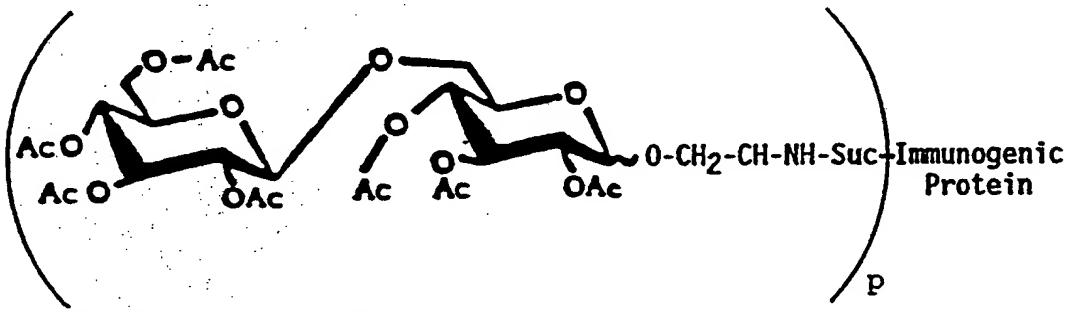
(a) the lipid A analog/immunogenic carrier conjugate of claim 1; and

(b) a pharmaceutically acceptable carrier;

wherein said conjugate is present in an amount effective to induce active immunity to LPS in an animal.

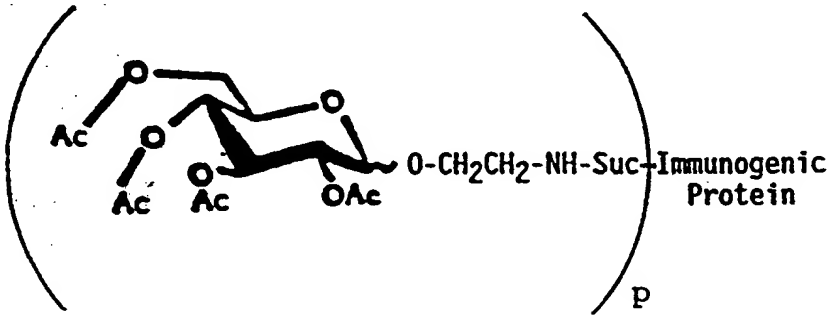
23. The vaccine of claim 22, further comprising an adjuvant.

24. The vaccine of claim 22, wherein said conjugate has the formula:



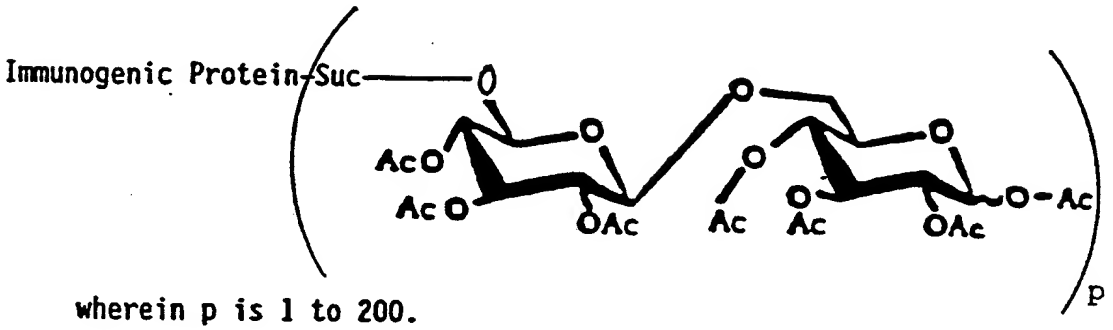
wherein p is 1 to 200.

25. The vaccine of claim 22, wherein said conjugate has the formula:



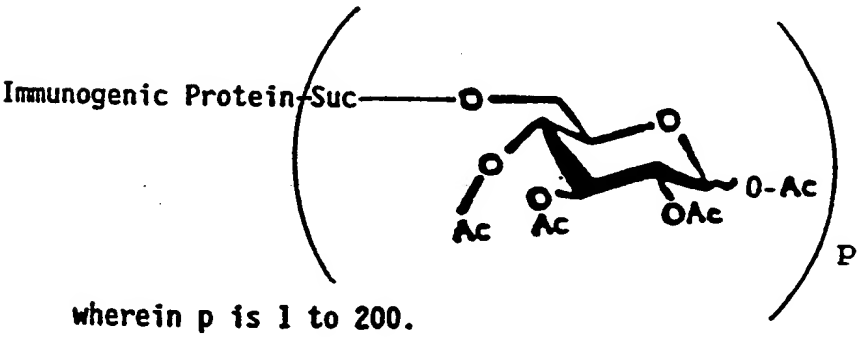
wherein p is 1 to 200.

26. The vaccine of claim 22, wherein said conjugate has the formula:



wherein p is 1 to 200.

27. The vaccine of claim 22, wherein said conjugate has the formula:



wherein p is 1 to 200.

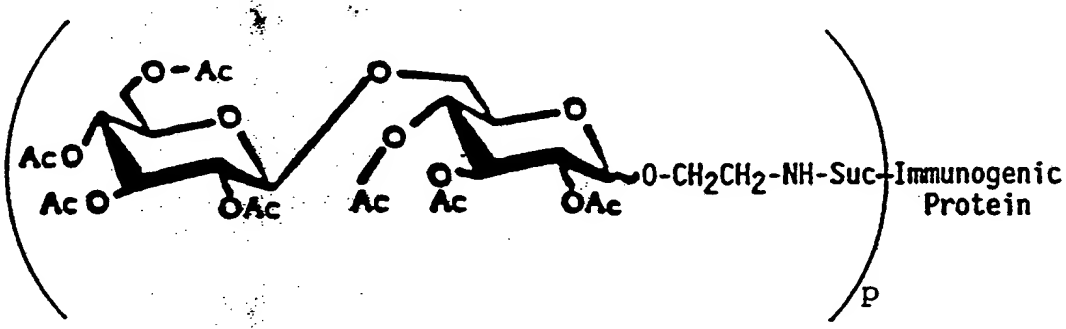
28. A method for treating or preventing septic shock in an animal comprising administering a pharmaceutical composition to an animal comprising:

(a) the lipid A analog/immunogenic carrier conjugate of claim 1; and

(b) a pharmaceutically acceptable carrier;
wherein said lipid A analog/immunogenic carrier conjugate is present in an amount effective to induce active immunity to LPS in an animal.

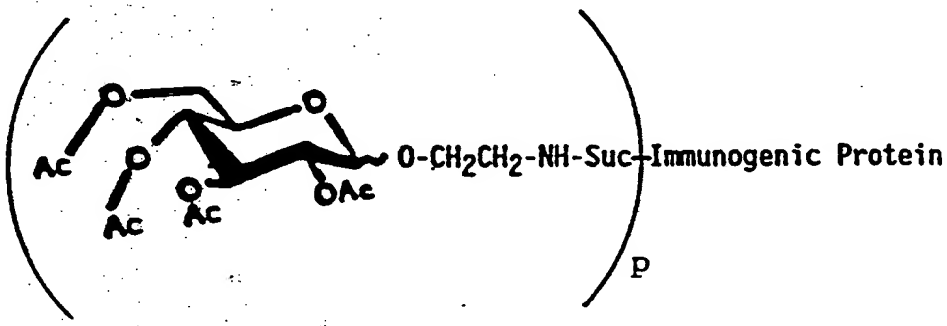
29. The method of claim 28, wherein said amount effective is 0.001 to 25.0 μg/kg body weight of said animal.

30. The method of claim 28, wherein said conjugate has the formula:



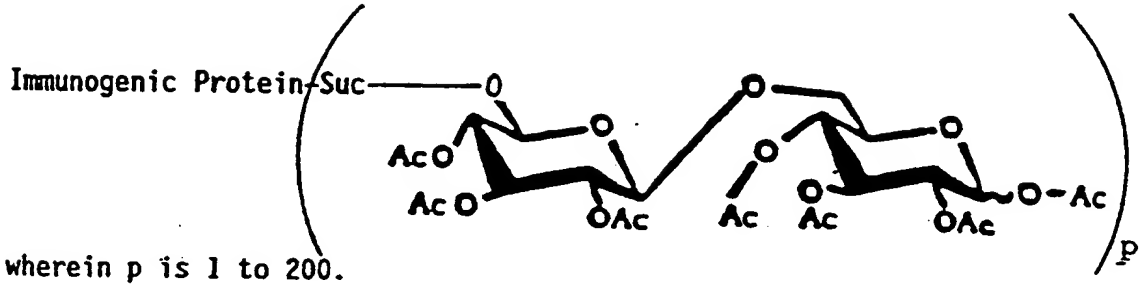
wherein p is 1 to 200.

31. The method of claim 28, wherein said conjugate has the formula:



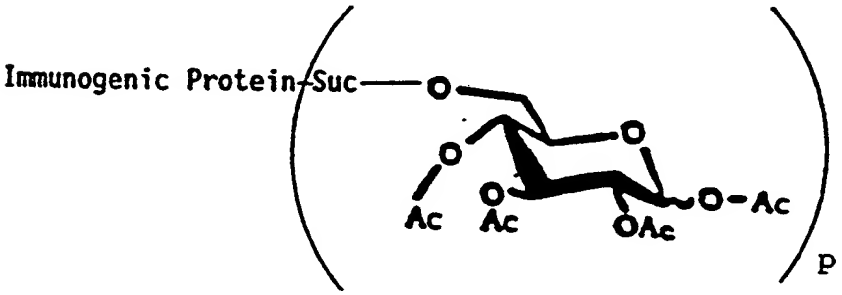
wherein p is 1 to 200.

32. The method of claim 28, wherein said conjugate has the formula:



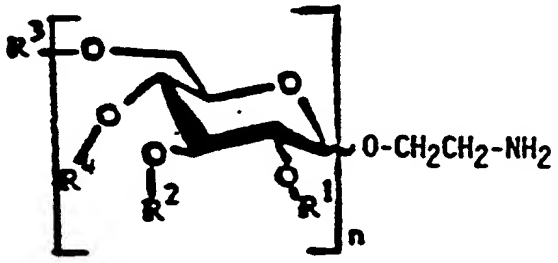
wherein p is 1 to 200.

33. The method of claim 28, wherein said conjugate has the formula:



wherein p is 1 to 200.

34. A compound having the formula:



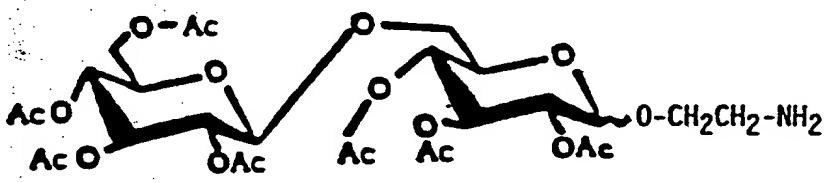
wherein n=2,

R^1 and R^3 are the same or different and selected from the group consisting of hydrogen, C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups;

R^2 is selected from the group consisting of C_2-C_{18} acyl groups, 3-hydroxy C_3-C_{18} acyl groups, and 3-(C_2-C_{12}-acyloxy)-C_3-C_{18} acyl groups; and

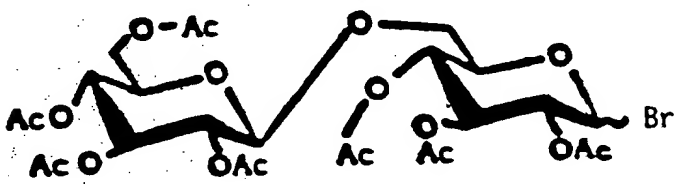
R^4 is hydrogen, a C_2-C_{18} acyl group or a phosphate group.

35. A compound having the formula:



36. A compound having the formula:

37. A compound having the formula:



38. A method of prepaparing a hyperimmune serum comprising administering the lipid A analog/immunogenic carrier conjugate of claim 1 to an animal and collecting the hyperimmunized serum.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04087

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 39/02, 39/095, 39/102, 39/104, 39/108

U.S.CL: 424/92, 88; 530/404, 405, See attachment

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/88,92; 530/404, 405, 406, 410, 377,363, 380, 395; 536/4.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

DIALOG AND APS COMPUTER SEARCH FILES

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
P,Y	US, A, 4,894,229 (Polson et al) 16 January 1990, See columns 3, 4,17 and 18.	38
P,Y	US,A, 4,902,506 (Anderson et al) 20 February 1990, See column 5.	1-11, 14-20 22,23,28,29 38
Y	US, A, 4,844,894, (Ribi) 04 July 1989, See column 1 and 2.	38
Y	US, A, 4,695,624 (Marburg et al) 22 September, 1987, See columns 3, 4, 7 and 8.	1-11,14-20, 22,23,28,29, 38
Y	Methods in Enzymology, volume 93, issued 1983, T.I. Ghose et al, "Preparation of Antibody-linked Cytotoxic Agents" Pages 280-333, see page 324.	1-11,14-20, 22,23,28,29 38
Y	Reviews of Infectious Diseases, volume 6, No. 4, issued July-August 1984, G. Galanos et al, "Immunogenic Properties of Lipid A"	1-11,14-20, 22,23,28,29, 38

See Attachment

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
11 October 1990	27 NOV 1990
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Kay K. Kim, Ph.D.

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
	pages 546-552, see pages 546, 547 and 550	
Y	Infection and Immunity, Volume 17, No. 1, issued July 1977, MA Johns et al, "Immunization with R Mutants of Salmonella minnesota II. Serological Response to Lipid A and the Lipopolysaccharide of Re Mutants" pages 9-15, see pages 9 and 10.	38
Y	Carbohydrate Research, Volume 90, issued 1981, M. Kiso et al, "Synthesis and immunoadjuvant activities of novel N-acylmuramoyl dipeptides related to the lipid A constituent of the bacterial lipopolysaccharide", pages C8-C11, see pages C8 and C9.	1-11,14-20,22,23, 28,29,38
Y	R.H Morrison et al "ORGANIC CHEMISTRY" published 1973 by Allyn and Bacon, Inc. (Boston), See pages 1094 and 1095.	1-11,14-20,22,23 28,29,38
Y	Infection and Immunity, Volume 10, No. 6 issued December 1974, NA. Mullan et al, "Protection Against Gram-Negative Infections with Antiserum to Lipid A from Salmonella Minisota R595" pages 1195-1201, See pages 1195 and 1196.	1-11,14-20,22,23 28,29,38

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers..... . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. Claims 1-33 and 38 drawn to lipid A analog-carrier conjugates and methods of use thereof classified in classes 424 and 530, subclasses 92 and 88 and 404, 405, 406, and 395, respectively.

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-33 and 38, see Telephone practice

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment to PCT/ISA/210
I. Classification Of Subject Matter

IPC(5): 39/112, 39/385; C07K 17/06; C07H 11/04
U.S.CL: 406, 395; 536/4.1

Attachment to PCT/ISA/210
VI. Observations Where Unity of Invention Is Lacking

2. Claims 34-37, drawn to compounds, classified in class 536, subclass 4.1.

Detailed Reasons for Holding Lack of Unity of Invention:

The invention of Group II and Group I are related as mutually exclusive species in intermediate-final product relationship wherein there are no provisions in PCT Rule 13.2 for claims drawn to intermediate final product relationships. The intermediate product of the claimed invention is useful to make other than the final product immunogenic conjugate such as an affinity column matrix ligand , therefore the species are distinct.

Applicant is invited to pay for the search of the additional invention group. The fee for the each additional invention is $150.00.

This International Searching Authority will establish the international search report on those parts of the international application which relates to the invention first mentioned as set forth above and as represented by claim Nos. 1-33 and 38.

Applicant is advised that in accordance with PCT Rule 40.2, a protest for holding of lack of unity of invention can be filed only for Groups for which additional search fees were paid. Such protest must be filed within 15 day of the mailing of the Search Report (form 210).